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DEVELOPMENT OF A TOXICITY TEST SYSTEM USING PRIMARY RAT LIVER CELLS

JEFF INMON, ANDY STEAD, MICHAEL D. WATERS, AND JOELLEN LEWTAS[1]

Genetic Toxicology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711

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SUMMARY

A model in vitro rat liver parenchymal cellular toxicity system employing cells obtained by the in situ collagenase perfusion technique has been developed to detect potential liver toxicants. The initial evaluation of this test system was accomplished using cadmium chloride, chromium chloride, cobalt chloride, mercuric chloride, nickelous chloride, sodium arsenite, sodium selenite, and ammonium vanadate. Linear regression analysis of the dose response curves was used to determine the effective concentration at which the viability was reduced to 50% (EC_{50}). The relative toxicity of the compounds was as follows: Cd > V = As > Se > Hg > Cr = Co > Ni. Since several of the compounds with very similar EC_{50}s had significantly different dose response slopes, an additional parameter, lowest effective concentration tested (LECT) was employed to assess the relative toxicity. The LECT was determined using the Williams test and the relative toxicity of the compounds was found to be Cd = Se > V > As = Hg > Co > Cr = Ni. The primary objective in developing this rat liver cellular toxicity test system was to employ an in vitro test system utilizing metabolically active primary cells from a potential target organ. This study demonstrates the utility of this test system in determining the relative liver cell toxicity of a series of inorganic agents of differing toxicity.

Key words: liver; perfusion; trace metals; viability; primary culture; cellular toxicity.

INTRODUCTION

Liver parenchymal cells play an essential role in metabolism of both natural and synthetic compounds. Virtually every hepatic activity investigated to date is measurable in isolated liver parenchymal cells, although the level of activity may vary with reference to the liver in vivo, especially as a function of time in culture. Many of these activities remain near in vivo levels for several days, including albumin synthesis and secretion, gluconeogenesis from 3-carbon precursors, glycogen synthesis, responsiveness of the cells to insulin and glucagon, and inducibility of *p*-nitroanisole *o*-demethylase, a microsomal mixed-function oxygenase (1,2). Bile salt conjugation, uptake and storage of sulfobromophthalein, and heme catabolism to bilirubin all appear to be maintained near the expected in vivo level (1). One of the markers of differentiated function of hepatic cells in culture is the inducibility of tyrosine aminotransferase following addition of glucocorticoids such as dexamethasone to the cultures. This increase in enzyme specific activity is similar from the 2nd through the 4th d in culture (3,4) and provides a useful marker of liver cell function.

Rat liver parenchymal cells obtained by a modification of the in situ collagenase perfusion technique of Bonney (3) and Laishes and Williams et al. (5–7) have been evaluated in our laboratory as metabolically active primary cells for studying the cellular toxicity of chemicals (8,9). Perfusion and culture conditions have been selected to give high viability and cell yields.

This paper describes a model in vitro system to assess the relative cytotoxic properties of a series of inorganic metals and nonmetals that occur as

[1]To whom requests for reprints should be addressed at (MD-68) Genetic Toxicology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711.

environmental contaminants. Rat parenchymal cells obtained by liver perfusion are exposed in tissue culture for 20 h to chlorides of Hg^{+2}, Cd^{2+}, Ni^{2+}, Cr^{3+}, Co^{+2}, and to ammonium vanadate (NH_4VO_3), sodium selenite (Na_2SeO_3), and sodium arsenite (Na_2AsO_2).

Two different statistical methods were used to evaluate the relative cellular toxicity of this series of inorganics: (a) linear regression (10) to estimate the concentration of each compound that reduced cellular viability to 50% of control (EC_{50}) and (b) the Williams test (11,12), to determine the lowest effective concentration tested (LECT) which resulted in a significant decrease in viability when compared to controls.

Materials and Methods

Animals. Adult male Sprague-Dawley rats weighing 250 to 300 g (Charles River Breeding Lab, Wilmington, MA) were housed in a 12 h light/dark cycle animal room, maintained on Purina Lab Chow (Ralston Purina, St. Louis, MO), and provided water ad libitum. Hepatic parenchymal cells were isolated from the animal within 2 to 3 h after the initiation of the light cycle.

Chemicals. The following chemicals were used in this study: ammonium vanadate (Pfaltz and Bauer, Inc., Flushing, NY), cadmium chloride (Matheson, Coleman and Bell, Norwood, OH), nickelous chloride (The International Nickel Co., Inc., Suffern, NY), chrominum chloride (J. T. Baker Chemical Co., Phillipsburg, NJ), sodium selenite (Pfaltz and Bauer), mercuric chloride (K&K Laboratories, Inc., Plainview, NY), sodium arsenite (Fisher Scientific Co., Fairlawn, NJ), and cobalt chloride (Fisher Scientific Co.).

Rat parenchymal cell isolation. Liver parenchymal cells were isolated by a modification of the in situ perfusion method of Bonney (3) and Laishes and Williams et al. (5–7). Rats weighing 250 to 300 g were anesthetized by the inhalation of ether. The abdomen was opened and the inferior vena cava ligated and cannulated proximal to the renal vein. The inferior vena cava distal to the exit from the liver and proximal to the heart was clamped with forceps either above or below the diaphragm. Initial washout was then performed by perfusing the liver for 5 min at a rate of 20 ml/min with a chelating solution of 0.5 m*M* ethyleneglycol bis (β-aminoethyl ether)-*N,N'*-tetracetic acid (Sigma Chemical Co., St. Louis, MO) in Hanks' Ca^{2+}-Mg^{2+}-free balanced salt solution, supplemented with 0.05 mg/ml gentamicin (Schering Corporation, Kenilworth, NJ). Immediately after beginning the initial washout, the hepatic portal vein was severed so that the effluent from the liver flowed freely into the abdominal cavity. Upon completion of the initial washout, the liver was then perfused at a rate of 20 ml/min for 10 min with 0.05% Type I collagenase (Sigma Chemical Co., St. Louis, MO, 146 U/mg) in Leibovitz medium (L15) (GIBCO, Grand Island, NY) supplemented with 0.05 mg/ml gentamicin. All perfusion solutions were maintained at 37° C in a water bath and continuously equilibrated with 5% CO_2 and air throughout the procedure. The collagenase solution was prepared fresh each day and sterilized by passage through washed Millipore filters (0.22 μm pore size).

At the termination of the perfusion, the liver was excised, trimmed, and placed in 50 ml of the collagenase solution in a 100 × 20 mm culture dish. The liver cells were detached by combing with an aluminum tooth dog comb. The detachment was completed after 5 min, leaving a residue of white fibrous tissue. The detached cell suspension was filtered through size 62 and 250 micron nylon mesh monofilament (Small Parts, Inc., Miami, FL) and centrifuged at 50 ×*g* for 5 min at 4° C. The supernatant was decanted, leaving the cells in 2 to 4 ml of solution. Fresh complete L15 medium (Leibovitz supplemented with 1 mg/ml glucose, 10% fetal bovine serum, 0.05 mg/ml gentamicin) is added to the cells. Simultaneous determination of cell viability by trypan blue dye exclusion and cell number per milliliter of cell suspension was performed by the method of Waters et al. (13) using the Model 6301 cytograph (Biophysics System, Mahopac, NY). The cytograph uses a ruby laser to generate a finely collimated light beam which interacts with single cells flowing through a cuvette. Opposite the beam there are two sets of detectors for measurement of light absorption and light scattering. The cytograph permits quantitation of cell number, size, viability, and various cytochemical effects, on a per cell basis, and correlation with biochemical changes as a function of concentration and time. The recovery of liver cells averaged 327×10^6 cells/rat with the initial viability being 77.9%, which was significantly improved to 95.7% by a medium change approximately 1.5 h after plating the dissociated liver.

Chemical treatment and experimental design. Cells were seeded at 1.5×10^6 cells/25 cm^2 tissue

culture flask (Falcon Plastic, Oxnard, CA) in a total volume of 5 ml complete L15 medium. After the 1.5 h attachment and the removal of nonattached cells, the compound to be studied was added directly into the flask with the complete L15 medium. The same volume of solvent was added to the control cultures free of test agent.

Each metal was prepared as a 100 m*M* stock solution in deionized water, filtered through 0.22 μm pore size filters. Aliquots of the stocks were diluted in culture medium just prior to testing. The metals were pH adjusted to 7.2 and aliquots of each metal and media tested were analyzed for actual metal ion concentration using the Perkin-Elmer Model 306 atomic absorption spectrophotometer.

A preliminary range finding test was first conducted at 0.001, 0.01, 0.1, 1.0 and 10 m*M*. Based on the preliminary range finding test data, three separate concentration response tests were conducted at seven concentrations. Triplicate flasks were used at each concentration tested. The concentrations were selected so that the initial portions of the linear dose response region were adequately tested as required to use the Williams test (*see* Statistical Methods).

A 20-h exposure period was selected to permit maximum overnight exposure time for development of cytotoxic effects in experimental cultures. After the 20-h exposure period, the media containing unattached cells were decanted and retained separately in culture tubes. The remaining attached cells were dissociated using 0.25% trypsin + EDTA for 3 to 4 min with visual observation. The dissociated cells were then combined with the unattached cells in complete L15 medium with serum, and placed on ice.

Trypan blue, freshly diluted with 0.85% saline to a final concentration of 0.01% was added to the 7 ml cell suspension for the determination of cell viability.

The viability of control cells was routinely 86% after 20 h in culture. Cell number and viability were obtained directly from the cytograph and expressed as percent of control.

Statistical methods. As the dose-response pattern generally differs from compound to compound, a series of mathematical transformations of the response variable (percent viability expressed as percent of control) or the independent variable (concentration) or both were performed to select the simple linear regression model. An EC_{50} and a corresponding 95% confidence interval for each tested compound were estimated using the selected regression model (10).

To find the LECT for each compound, a statistical procedure developed by Williams (11,12) was employed. To use this procedure, it was first necessary to run an analysis of variance on the raw viability data, considering each dose level (including control) as a treatment, to obtain an estimate of the error mean square per experimental unit. Then, assuming that cell viability decreases monotonically with increasing dose, maximum likelihood estimates of the mean response at each of the tested concentrations were obtained. A modified *t*-statistic based on these estimates was computed to test whether the mean response at a particular dose was significantly lower than the control response. Applying this test in sequential fashion, the lowest concentration at which there was evidence of a response could be determined.

The standard deviation and the coefficient of variation were determined as described by Snedecor and Cochran (14).

Results and Discussion

The recovery of liver cells averaged 327 × 10^6 cells/rat with the initial viability being 77.9 ± 4.6%. After 1.5 h incubation at 37° C, the nonattached cells were removed by fluid changing, resulting in an average attachment efficiency of 52.5 ± 3.5% (Table 1). The remaining attached cells showed a significantly improved viability of 95.7 ± 1.4% and rounded morphol-

TABLE 1

Recovery of Hepatocytes, Viability, and Attachment Efficiency

	Rat Weight	Total Cells/Rat × 10^6	Initial %V	1.5 h %V	21.5 h %V	% Attachment at 1.5 h
Mean[a]	268.00	327.00	77.90	95.70	86.40	52.50
Min	235.00	244.00	71.00	94.00	84.00	47.00
Max	300.00	474.00	85.00	98.00	88.00	55.00
SD	22.26	79.52	04.65	01.42	01.58	03.50
Coeff Var %	08.31	24.32	05.97	01.48	01.83	06.67

[a] The above data represent the mean of 10 separate perfusions.

ogy (Fig. 1*a*). After 4 h in culture, the cells began to flatten out until contact was made with neighboring cells, resulting in sectional monolayers (Fig. 1*b*). At 21.5 h in culture, the cells exhibited more typical liver parenchymal morphology (Fig. 1*c*), however the cellular viability was reduced somewhat to 86 ± 1.58% (Table 1).

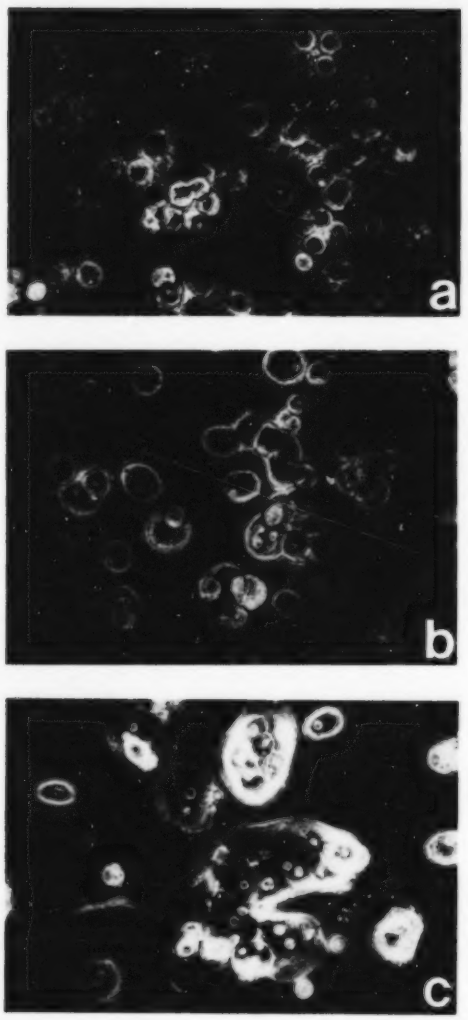

FIG. 1. *a*, Phase contrast ×250. Living adult liver parenchymal cells 1.5 h after plating dissociated liver; *b*, Phase contrast ×250. Living adult liver parenchymal cells 4 h after plating dissociated liver. The cells have flattened out and are beginning to form sectional monolayers; *c*, Phase contrast ×250. Living adult liver cells 21.5 h after plating dissociated liver. The cells have formed sectional monolayers and are cuboidal in shape. Many of the parenchymal cells are binucleated which is typical of parenchymal cells in vivo.

A primary liver cell toxicity test system was developed by exposing the cultured liver cells after the initial 1.5 h fluid change to fresh media with increasing concentrations of the chemical agent. The effect of the eight inorganic salts on viability is shown in Fig. 2.

Determination of cellular viability indicated that Cd, V, As, and Se salts were the most toxic elements evaluated, exhibiting EC_{50}s between 0.01 and 0.10 m*M*. Mercury showed less toxicity, with an EC_{50} of 0.15 m*M*. Chromium, Co, and Ni salts were the least toxic elements tested, with similar EC_{50}s between 2 and 4 m*M*. The EC_{50} for each compound tested was estimated from the linear regression analysis shown in Fig. 3. The EC_{50}s for V, As, and Se are similar (V, 0.042; As, 0.044, Se, 0.072) as are the EC_{50}s for Cr and Co (Cr, 2.196; Co, 2.512). However, the slope of the regression lines for As, V, and Se as well as for Cr and Co are much different. To quantitate the different toxicity of the compounds that shared similar EC_{50}s, the Williams test was employed. The Williams test detected the lowest concentration tested over the entire linear regression line (Fig. 3), which resulted in a significant decrease in viability from that exhibited by control.

In ranking the relative toxicity of this series of inorganic compounds, by EC_{50}s alone, the ranking would have been in the following order with decreasing toxicity: Cd > V = As > Se > Hg > Cr = Co > Ni (Table 2). By evaluating the LECT values, the ranking would be in the following order: Cd = Se > V > As = Hg > Co > Cr = Ni (Table 2). Additional evaluation of the lowest effective concentration tested (LECT) provided evidence that several elements (e.g., Se and Co) are more toxic at low doses than would be expected, based on determination of the EC_{50} alone, and other elements (e.g., As) are less toxic at low doses.

The combined use of these two toxicity parameters, the EC_{50} and LECT, provides a model approach applicable to other in vitro and in vivo toxicity test systems. The use of linear regression analysis of toxicity dose response curves to determine the lethal dose for 50% of the population (LD_{50}) or the effective concentration which reduces a parameter (e.g., cellular viability) by 50% (EC_{50}) has traditionally been used to provide a means of comparing the toxicity of a series of agents. The additional determination of the

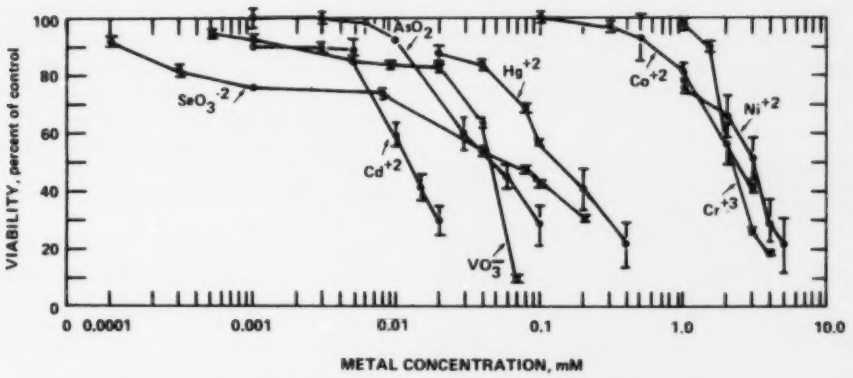

FIG. 2. Effect of metallic chlorides on viability of rat hepatic parenchymal cells.

lowest effective concentration tested (LECT) requires the testing of additional concentrations of agents at low doses and provides a method of ranking the toxicity of agents at low doses. This approach is of more significance to the evaluation of the potential environmental effects of chemicals since it is desirable to determine the concentration of a chemical which does *not* cause an adverse biological effect. The toxicity responses of the series of inorganic compounds compared in this study clearly show several compounds that were comparatively more toxic at low doses than they were shown to be based on evaluation of the EC_{50} at higher doses.

The principal objective of this work was to develop an in vitro test system utilizing metabolically active primary cells from a potential target organ. Liver cells similar to those employed in this study have been shown to retain nearly all of the synthetic and metabolic activities present in liver in vivo (1,2). Comparison of the relative toxicity response in liver cells with that previously

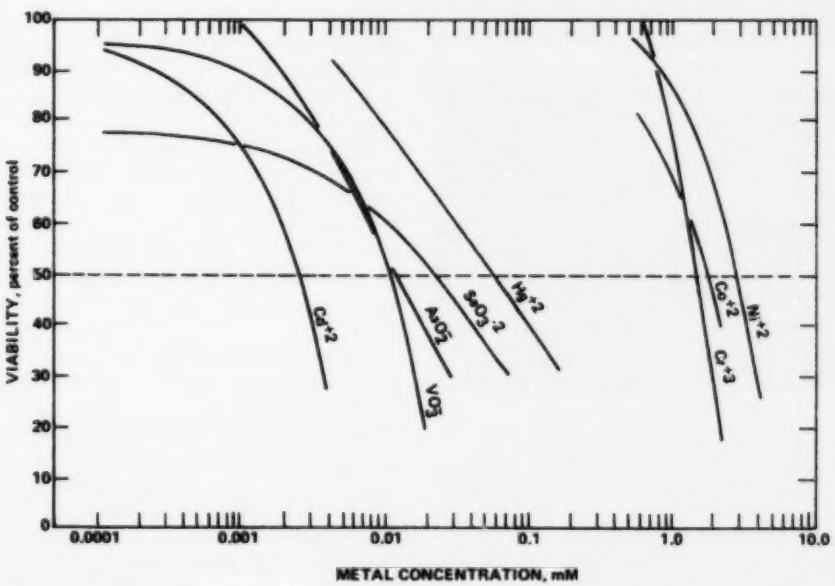

FIG. 3. Predicted response patterns of data shown in Fig. 2 from regression analyses of each metal.

TABLE 2

SUMMARY OF THE TOXICITY OF A SERIES OF INORGANICS

Ranking Based of EC_{50}[a] (m*M*)		Ranking Based on LECTs[b] (m*M*)	
1. Cd^{+2}	0.013 (0.010–0.017)	1. Cd^{+2}	0.001
2. VO_3^{-2}	0.042 (0.021–0.066)	2. SeO_3^{-2}	0.001
3. AsO_2^{-1}	0.044 (0.030–0.058)	3. VO_3^{-2}	0.0049
4. SeO_3^{-2}	0.072 (0.046–0.099)	4. AsO_2^{-1}	0.030
5. Hg^{+2}	0.159 (0.076–0.366)	5. Hg^{+2}	0.040
6. Cr^{+3}	2.196 (1.484–2.982)	6. Co^{+2}	0.500
7. Co^{+2}	2.512 (2.150–2.916)	7. Cr^{+3}	2.000
8. Ni^{+2}	3.578 (2.016–4.636)	8. Ni^{+2}	2.000

[a] The concentration of each ion that reduced cellular viability to 50% of control cultures. The 95% confidence limits are shown in parentheses.

[b] LECT which resulted in a significant decrease in viability for the treated versus the control cultures as determined by the Williams test.

reported by Waters et al. (15) for primary lung (alveolar) macrophages and WI-38 lung fibroblasts is shown in Table 3. Liver cells are generally more sensitive to the elements tested than the other two cell types, and therefore show lower EC_{50}s for each compound except mercuric chloride ($HgCl_2$).

The reason for the comparatively higher sensitivity of liver cells to many of these elements is not known. Factors potentially responsible for this sensitivity include (a) increased cellular permeability due to the collagenase treatment, (b) differences inherent in primary cells compared to cell lines adapted to culture, and (c) differences inherent in cell types (e.g., lung versus liver cells).

The high toxicity of Cd observed in liver cells suggests that the liver may be a target organ in cadmium toxicity in vivo. Cadmium is known to accumulate and induce functional alterations in the liver of animals exposed to cadmium (16). Since liver is also a major site of synthesis of the cadmium binding protein, metallothionein, cultured hepatocytes may be useful in studying the effect of induction of this protein by cadmium pretreatment and subsequent cadmium toxicity.

TABLE 3

CONCENTRATIONS (m*M*) OF METALLIC IONS CAUSING REDUCTION IN VIABILITY TO 50% (EC_{50}) IN CULTURED LIVER PARENCHYMAL CELLS, RABBIT ALVEOLAR MACROPHAGES AND WI-38 CELLS AFTER 20 H

Ion	Liver Cells	Macrophages	WI-38
Cd^{+2}	0.013	0.099	0.270
VO_3^{-2}	0.042	0.234	0.275
SeO_3^{-2}	0.072	4.000	—
Hg^{+2}	0.159	0.050	—
Cr^{+3}	2.196	5.480	10.530
Ni^{+2}	3.578	4.170	3.420

Selenium was also significantly more toxic to liver cells than the lung macrophages. Although little inhalation toxicology data are available to explain the apparent resistance of these lung cells to selenium, it is known that liver cells are able to metabolize sodium selenite to demethylselenide, which is eliminated via exhalation in the lung (17). This suggests that lung cells may be less sensitive to selenium toxicity in general.

Cultured primary liver cells may also be useful in evaluating and elucidating the mechanism of trace element interactions known to occur both in vitro (18) and in vivo (19,20). The test system described here utilizes liver cells from animals fed a diet containing essential trace elements and cultured in media containing fetal bovine serum. Under these conditions, the liver cells should be in a normal trace element status. The final media contains zinc (0.0015 m*M*), selenium (0.0001 m*M*), and copper (0.0016 m*M*) in concentrations below that necessary to cause significant antagonistic interactions (18) with the series of inorganic compounds evaluated in this paper.

The use of primary cultured liver cells in evaluating the potential liver toxicity of chemicals has specific advantages in many cases when liver specific metabolism plays an important role in the ultimate toxicity. This is particularly true in the evaluation of organic agents. This liver cell toxicity system is now being extended to the evaluation of both organic and inorganic chemicals using the culture, exposure, and statistical methodologies described here with the measurement of additional functional and biochemical changes.

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